Mannitol Protects against Oxidation by Hydroxyl Radicals[1]

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Hydroxyl radicals may be responsible for oxidative damage during drought or chilling stress. We have shown that the presence of mannitol in chloroplasts can protect plants against oxidative damage by hydroxyl radicals (B. Shen, R.G. Jensen, H.J. Bohnert [1997] Plant Physiol 113: 1177–1183). Here we identify one of the target enzymes that may be protected by mannitol. Isolated thylakoids in the presence of physiological concentrations of Fe^{2+} generated hydroxyl radicals that were detected by the conversion of phenylalanine into tyrosine. The activity of phosphoribulokinase (PRK), a thiol-regulated enzyme of the Calvin cycle, was reduced by 65% in illuminated thylakoids producing hydroxyl radicals. Mannitol (125 mM) and sodium formate (15 mM), both hydroxyl radical scavengers, and catalase (3000 units mL^{-1}) prevented loss of PRK activity. In contrast, superoxide dismutase (300 units mL^{-1}) and glycine betaine (125 mM) were not effective in protecting PRK against oxidative inactivation. Ribulose-1,5-bisphosphate carboxylase/oxygenase activity was not affected by hydroxyl radicals. We suggest that the stress-protective role of mannitol may be to shield susceptible thiol-regulated enzymes like PRK plus thioredoxin, ferredoxin, and glutathione from inactivation by hydroxyl radicals in plants.

ROS, including superoxide, H_2O_2, and hydroxyl radicals, are toxic and their action can result in oxidative damage of proteins, membrane lipids, and other cellular components (Halliwell and Gutteridge, 1990; Asada, 1994). Oxidative damage of proteins by ROS has been well characterized. ROS can attack amino acid residues in proteins, especially Tyr, Phe, Trp, Met, and Cys, and can form carbonyl derivatives (Stadtman, 1992). Moreover, ROS promote intra- and intermolecular cross-linking, such as -S-S-bonding and protein fragmentation. Such modifications mark proteins for degradation (Stadtman, 1992). The damage of membranes by ROS has been documented by increased formation of malondialdehyde, a product of lipid peroxidation, during oxidative stress (Smirnoff, 1993; Wise, 1995). In addition, a physiological protective mechanism that can limit the spread of pathogens uses the production of ROS to initiate localized cell death and a hypersensitive response to pathogen infection (Tenhaken et al., 1995).

Production of ROS is an unavoidable process in illuminated chloroplasts. Superoxide is mainly produced from photoreduction of oxygen via the reduction of oxygen by PSI (Mehler and Brown, 1952; Robinson, 1988). Most H_2O_2 in chloroplasts is then produced through disproportionation of superoxide by SODs. Additionally, hydroxyl radicals are produced in the Haber-Weiss or Fenton reactions through the interaction of H_2O_2 and superoxide or directly from H_2O_2 in the presence of transition metals, such as Fe^{+2} and Cu^{+2} (Halliwell and Gutteridge, 1990). Removal of these ROS is essential for chloroplast function. Several enzyme systems, as well as the presence of ascorbate and glutathione, have evolved to scavenge ROS. Superoxides are converted to H_2O_2 by SODs and are broken down to water by the ascorbate peroxidases. In contrast to superoxide and H_2O_2, enzymes that scavenge hydroxyl radicals have not been detected (Asada and Takahashi, 1987; Asada, 1994).

The targets most susceptible to damage by ROS in illuminated chloroplasts are not known in detail, but it is most likely that ROS affect both the photosynthetic electron transport machinery and Calvin cycle enzymes. It also seems that, compared with the effects of ROS on stromal enzymes, thylakoid proteins and electron carriers are less sensitive to superoxide and H_2O_2 (Asada, 1994). H_2O_2 can inactivate photosynthesis in isolated intact chloroplasts at low concentrations. The rate of CO_2 fixation, for example, was inhibited by 50% following the addition of 10 μM H_2O_2 to intact chloroplasts and the addition of catalase partially restored CO_2 fixation (Kaiser, 1976). The SH-enzymes in the Calvin cycle are known to be sensitive to H_2O_2 and their activities are regulated by oxidation/reduction of thiol groups via the Fd/thioredoxin system, resulting in day/night changes in activity (Kaiser, 1979; Tanaka et al., 1982a, 1982b; Buchanan, 1991). The reduction of thioredoxin is directly linked to PSI via Fd (Buchanan, 1991). Based on the sensitivity of SH-enzymes to oxidants, they could be the primary targets of ROS in the stroma.

We were interested in the functions of polyols, which increase in a number of species during environmental stress (Ahmad et al., 1979; Bohnert and Jensen, 1996), especially in the possible function of mannitol as a hydroxyl radical scavenger. Our previous results indicated that targeting mannitol biosynthesis to chloroplasts in transgenic tobacco (*Nicotiana tabacum*) resulted in increased radical-scavenging capacity and thus increased resistance to oxidative stress (Shen et al., 1997). However, target molecules

Abbreviations: PRK, phosphoribulokinase (EC 2.7.1.19), ROS, reactive oxygen species, RuBP, ribulose-1,5-bisphosphate; SOD, superoxide dismutase (EC 1.15.1.1), SH-enzyme, thiol-regulated enzyme.

that mannitol may protect have not been identified. In this study we utilized isolated thylakoids to produce hydroxyl radicals that were detected by a Phe-Tyr reaction. In this system mannitol was tested to determine whether its presence could protect PRK against oxidative inactivation by hydroxyl radicals.

MATERIALS AND METHODS

Isolation of Thylakoids

Thylakoids were isolated from tobacco (*Nicotiana tabacum*) leaves according to the procedures of Paterson and Arntzen (1982) with some modifications. Leaves (20 g) were homogenized in 100 mL of extraction buffer containing 400 mM Gly betaine, 50 mM Tricine-NaOH, pH 7.8, and 5 mM ascorbate, were filtered through a 50-μm nylon mesh, and then were centrifuged at 1000g for 8 min. The resulting pellet was washed with washing buffer (20 mM potassium phosphate buffer, pH 7.8, 10 mM NaCl, and 5 mM MgCl$_2$) and resuspended in the same washing buffer at 0.5 mg chlorophyll mL^{-1}. Chlorophyll content was determined as described previously (Arnon, 1949).

Analysis of Hydroxyl Radicals Production

Hydroxyl radicals were produced from either a Fenton reaction or an isolated thylakoid system. The Fenton reaction contained 100 μM FeCl$_3$, 100 μM ascorbate, 100 μM EDTA, 25 mM potassium phosphate buffer, pH 7.4, and 1 mM H$_2$O$_2$. In the isolated thylakoid system, H$_2$O$_2$ was replaced by thylakoids. The reaction mixture contained 50 μg chlorophyll mL^{-1} thylakoids, 100 μM FeCl$_3$, 100 μM ascorbate, 100 μM EDTA, and 25 mM potassium phosphate buffer, pH 7.8. The mixture was incubated at 25°C in light (450 μmol m^{-2} s^{-1}) for 1 h and the reaction was stopped by adding 0.6 mL of ethanol:chloroform:water (12:5:3, v/v). To measure production of hydroxyl radicals in the reactions, 5 mM Phe was added and Tyr formation was determined (Kaur et al., 1988; Kaur and Halliwell, 1994).

Tyr produced from Phe in a Fenton reaction was separated directly by HPLC without prepurification. Tyr produced in a thylakoid mixture was purified using a cation-exchange column as described previously (Lazarus, 1973; Walton et al., 1991). The ethanol/water phase of the extract was loaded on a 0.5-mL cation-exchange column (AG50W-X4, 200–400 mesh, Bio-Rad). The column was prewashed with 5 mL of 1 N HCl and 5 mL of 0.2 N NaOH, followed by 5 mL of 1 N HCl. After the sample was loaded, the column was washed with 5 mL of 0.01 N HCl and then 5 mL of water. The Tyr was eluted by adding 2 mL of 0.2 N NaOH. The first 0.5 mL of eluate containing no Tyr was discarded. The eluate was neutralized with HCl, filtered through a nylon filter, and vacuum evaporated to 800 μL. The purified sample (100 μL) was then loaded on the HPLC. HPLC separation and analysis of Tyr was carried out on an IonPac CS14 column with a IonPac CG14 guard column (Dionex, Sunnyvale, CA). The elutant was 20 mM methane sulfonic acid in 10% methanol at a

flow rate of 1.0 mL min^{-1}. Tyr was detected with a UV detector at 278 nm.

Enzyme Activity Assay

PRK was measured based on production of RuBP. Spinach PRK (Sigma) was added to an isolated tobacco thylakoid mixture containing 50 μg chlorophyll mL^{-1} thylakoids, 100 μM FeCl$_3$, 100 μM ascorbate, 100 μM EDTA, 25 mM potassium phosphate buffer, pH 7.8, 20 mM MgCl$_2$, 1 mM ATP, and 0.1 unit of PRK. The reaction was started by adding 1 mM ribulose-5-phosphate and incubated at 25°C in 450 μmol m^{-2} s^{-1} light or in darkness for 1 h; the reaction was stopped by adding five drops of cation-exchange resin (AG50W-X4) in the H$^+$ form. Addition of cation-exchange resin lowered the pH to less than 2.0 and denatured the enzyme. The reaction mixture was centrifuged at 10,000g for 5 min. The supernatant was neutralized and used for RuBP measurement. The amount of RuBP was measured upon formation of [^{14}C]P-glycerate following reaction with Rubisco. To eliminate any PRK contamination in the Rubisco preparation, a recombinant cyanobacterial Rubisco purified from *Escherichia coli* was used. The reaction mixture contained 50 mM Hepes, pH 8.0, 10 mM MaCl$_2$, 10 mM KH^{14}CO$_3$ (2 Ci mol^{-1}), 0.05 unit of Rubisco with addition of 50- to 100-μL samples. The mixture was incubated at 25°C for more than 30 min to allow all RuBP to be used. All ^{14}CO$_2$ left in the reaction was removed by addition of 1 mL of 1 N HCl. After the sample was heat-dried at 80°C, the acid-stable radioactivity was determined by liquid scintillation counting. The amount of RuBP was calculated based on the amount of ^{14}C fixed. Standard RuBP was used as a control.

RESULTS

Production of Hydroxyl Radicals in Isolated Thylakoids

Isolated thylakoids were illuminated to produce hydroxyl radicals in the presence of Fe. Oxygen is photoreduced to superoxide, which is converted to H$_2$O$_2$ by SOD (Asada and Takahashi, 1987; Robinson, 1988). In the presence of Fe, hydroxyl radicals are then produced from H$_2$O$_2$ via a Fenton reaction. Production of hydroxyl radicals was monitored by using a Phe-Tyr-detecting system (Kaur and Halliwell, 1994). Phe was attacked by hydroxyl radicals and three isomeric Tyrs were produced, which were separated and analyzed by HPLC (Fig. 1). In white light (450 μmol m^{-2} s^{-1}), Tyr formation with isolated thylakoids increased, depending on thylakoid amount, and saturated at approximately 25 μg chlorophyll mL^{-1} (Fig. 2A). Without the addition of Fe, Tyr was formed at approximately 25% of the maximum rate (Fig. 2B). This rate of Tyr formed may be due to trace amounts of Fe released from the thylakoids. The concentration of Fe required for hydroxyl radical production was low. At 10 μM FeCl$_3$ the production of hydroxyl radical reached the maximum rate. Production of Tyr increased linearly with time (Fig. 2C). Based on these parameters, the conditions for the following experiments were chosen.
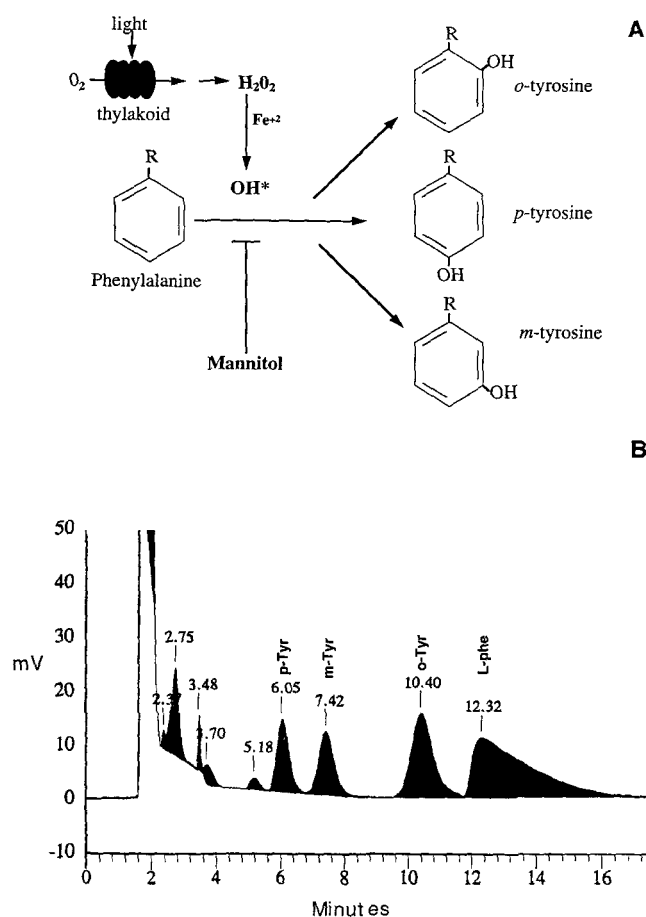
Figure 1. Production of hydroxyl radicals by isolated thylakoids. A, Diagram of the hydroxyl radical production and detection system. B, HPLC separation of the three Tyr isomers.

Mannitol Scavenges Hydroxyl Radicals in Vitro

In the Fenton reaction mannitol competes with Phe for hydroxyl radicals, resulting in inhibition of Tyr formation. The percentage of inhibition was dependent on the concentration of mannitol. For example, 20 mM mannitol inhibited Tyr formation by approximately 60%. In contrast to mannitol, another well-known, compatible solute, Gly betaine, up to 80 mM, did not inhibit Tyr formation (Fig. 3), which is consistent with previous results (Smirnoff and Cumbes, 1989). In isolated thylakoids only trace amounts of Tyr were found when the system was incubated in darkness, suggesting that hydroxyl radical formation required light-driven electron transport. DCMU inhibited electron transport from PSII to PSI and also inhibited Tyr formation by more than 95% during illumination (data not shown). In the light production of hydroxyl radicals led to a significant increase in Tyr formation. Catalase consumed H_2O_2, thus preventing Tyr formation. Mannitol inhibited Tyr formation by approximately 80% at a concentration of 125 mM, whereas the same concentration of Gly betaine had little effect on Tyr formation. Addition of SOD did not reduce and in some experiments even increased Tyr for-

mation. Methylviologen, which stimulated superoxide production in vivo, did not increase Tyr formation in this system (Fig. 4).

Mannitol Protects PRK against Oxidative Inactivation by Hydroxyl Radicals

H_2O_2 inactivates SH-enzymes in chloroplasts. This inactivation may be attributed to direct oxidation of SH groups by H_2O_2 or, alternatively, indirect oxidation by hydroxyl radicals formed from H_2O_2 via a Fenton reaction. To test whether hydroxyl radicals are involved in oxidative inactivation of PRK, a dithiol-regulated enzyme, PRK, was added to an isolated thylakoid system and its activity was
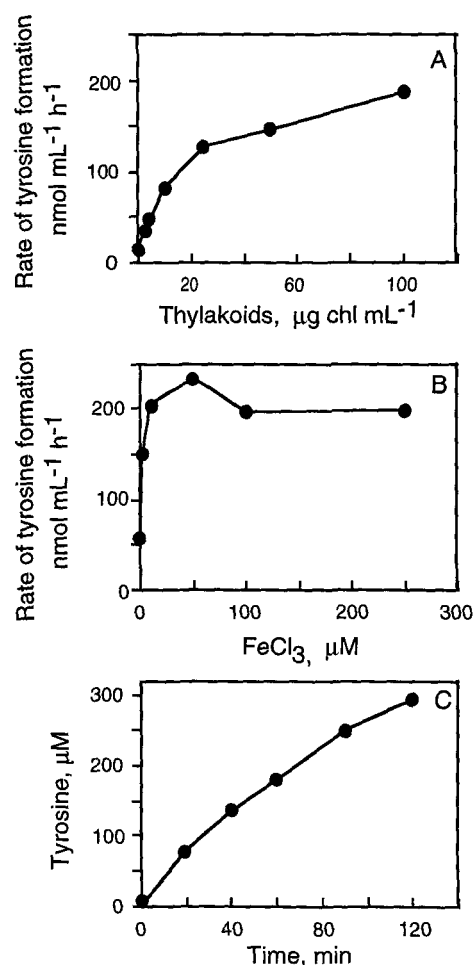

Figure 2. Effect of thylakoid and Fe concentration on Tyr formation and time course of Tyr formation in isolated thylakoids. A, Along with different thylakoid concentrations the reaction contained 100 μM $FeCl_3$, 100 μM ascorbate, 100 μM EDTA, and 5 mM Phe in 25 mM potassium phosphate buffer, pH 7.8. The mixture was illuminated at 25°C with 450 μmol m^{-2} s^{-1} white light and the Tyr's produced were determined by HPLC. B, With varying amounts of $FeCl_3$, 50 μg chlorophyll (chl) mL^{-1} thylakoids and other conditions as in A, Tyr production was determined by HPLC. C, Time course of Tyr formation with 100 μM $FeCl_3$, 50 μg chlorophyll mL^{-1} thylakoids, and conditions as in A. The Tyr concentration in all instances measured the sum of the three Tyr isomers.
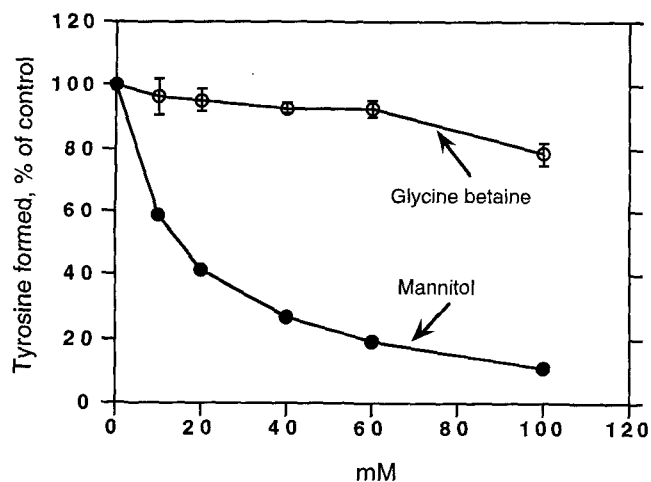
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Figure 3. Mannitol inhibits Tyr formation in a Fenton reaction. The reaction mixture contained 5 mM Phe, 25 mM potassium phosphate buffer, pH 7.5, 0.1 mM $FeCl_3$, 0.1 mM ascorbate, 0.1 mM EDTA, 1 mM H_2O_2, 100 mM mannitol, or 100 mM Gly betaine. The reaction was incubated at 25°C for 90 min and the Tyr amount was measured by HPLC. Values are the means ± SE of three measurements.

measured. When incubated in darkness, PRK retained relatively high activity. Addition of mannitol, Gly betaine, formate, SOD, or catalase did not affect its activity (Fig. 5). When incubated in the light, however, the activity of PRK was reduced by approximately 65%. Addition of 125 mM mannitol completely prevented the loss of PRK activity. Another hydroxyl radical scavenger, sodium formate (15 mM), showed the same effect. Equally important, addition of catalase reduced production of hydroxyl radicals by eliminating H_2O_2 and protected PRK against oxidative inactivation. Finally, DTT provided an SH-substrate for hydroxyl radicals (Halliwell, 1981) and also protected PRK activity. Gly betaine (Smirnoff and Cumbes, 1989) and SOD, which do not react with H_2O_2 or scavenge hydroxyl
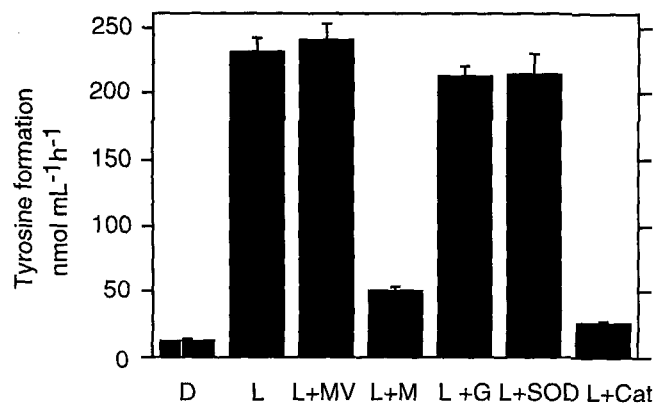
radicals, could not protect PRK against oxidative inactivation (Fig. 5). The results indicated that oxidative inactivation of PRK by H_2O_2 in chloroplasts most likely is mediated by the formation of hydroxyl radicals. As a control of enzymes not thiol regulated, Rubisco protein was added to the thylakoid preparations. Its activity was not affected by hydroxyl radicals (data not shown).

DISCUSSION

Among the ROS, hydroxyl radicals may be responsible for most of the oxidative damage during drought or chilling stress. Sufficient Fe is present in chloroplasts to sustain the Fenton reaction through which hydroxyl radicals can be generated (Whatley et al., 1951; Terry and Low, 1982; Kim and Jung, 1993). Our in vitro experiments indicated that 10 μM free Fe could sustain maximum production of hydroxyl radicals (Fig. 2). Increased concentrations of both H_2O_2 and free Fe have been observed in conditions of drought or cold stress, (Price and Hendry, 1991; Smirnoff, 1993; Moran et al., 1994; Wise, 1995). For example, free Fe and Cu increased 1.5- and 2.5-fold, respectively, under water stress and reached a concentration as high as 16 μM in the chloroplast stroma (Moran et al., 1994). This increase in the concentration of free transition metal ions may promote production of hydroxyl radicals via a Haber-Weiss reaction because 1 to 10 mM ascorbate in chloroplasts and 30 μmol superoxide mg^{-1} chlorophyll can reduce Fe^{3+} to Fe^{2+} (Asada, 1994; Moran et al., 1994). Moreover, the decline of activity of three SH-enzymes, sedoheptulose bisphosphatase, Fru bisphosphatase, and PRK, has been correlated with an increase of external Fe^{+2} concentrations in photoinhibited chloroplasts (Kim and Jung, 1993). Thus, oxidative damage observed in vivo as the result of environmental stress may be caused by increased production of

Figure 4. Mannitol inhibits Tyr formation in isolated thylakoids. The reaction mixture contained 50 μg chlorophyll mL^{-1} thylakoids, 100 μM $FeCl_3$, 100 μM ascorbate, 100 μM EDTA, 5 mM Phe, and 25 mM potassium phosphate buffer, pH 7.8, with the addition of the following and incubation for 1 h: D, darkness; L, 450 μmol m^{-2} s^{-1} light; L + MV, light plus 100 μM methylviologen; L + M, light plus 125 mM mannitol; L + G, light plus 125 mM Gly betaine; L + SOD, light plus 300 units mL^{-1} SOD; L + Cat, light plus 3000 units mL^{-1} catalase.
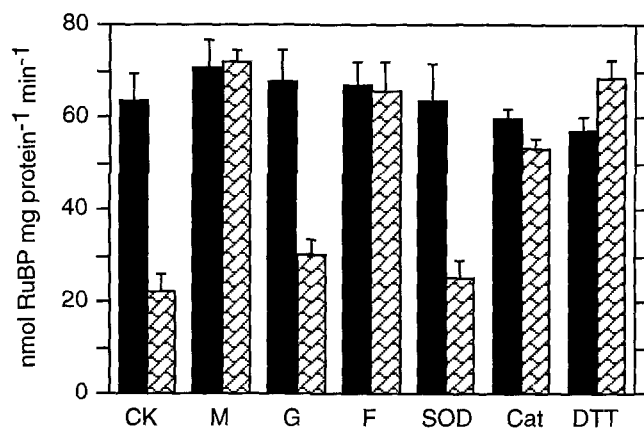

Figure 5. Mannitol protects PRK against oxidative inactivation by hydroxyl radicals. Spinach PRK was added to an isolated thylakoid mixture containing 50 μg chlorophyll mL^{-1} thylakoids, 100 μM $FeCl_3$, 100 μM ascorbate, 100 μM EDTA, 20 mM $MgCl_2$, and 1 mM ATP in 25 mM potassium phosphate, pH 7.8, with the following: CK, control; M, addition of 125 mM mannitol; G, addition of 125 mM Gly betaine; F, addition of 15 mM sodium formate; SOD, addition of 300 units mL^{-1} SOD; Cat, addition of 3000 units mL^{-1} of catalase; DTT, addition of 10 mM DDT.

hydroxyl radicals. In a preceding report (Shen et al., 1997), we investigated the capacity for radical scavenging when mannitol was engineered to be localized to chloroplasts. Targeting mannitol biosynthesis to tobacco chloroplasts enhanced the hydroxyl radical-scavenging capacity of the plants and increased their resistance to oxidative stress. We suggest that, in addition to their role in osmotic adjustment, the protective effect of polyols in stressed plants may be related to their ability to scavenge hydroxyl radicals (Ahmad et al., 1979; Adams et al., 1992; Tarczynski et al., 1993; Kishor et al., 1995).

SH-enzymes of the Calvin cycle have been found to be sensitive to H_2O_2 (Kaiser, 1976, 1979; Charles and Halliwell, 1980). Mannitol is known as a hydroxyl radical scavenger in vitro (Elstner, 1987; Smirnoff and Cumbes, 1989) and in vivo (Shen et al., 1997). To test whether mannitol can protect SH-enzymes from oxidative inactivation, we used an in vitro thylakoid system to mimic hydroxyl radical production in vivo. PRK served as the model for one of the SH-enzymes of the Calvin cycle. Mannitol or sodium formate, both of which are known hydroxyl radical scavengers, or catalase, which eliminates H_2O_2 before it can be converted to hydroxyl radicals, protected PRK against oxidative inactivation at physiological concentrations (Fig. 5). This suggests that hydroxyl radicals could be the active agent in illuminated chloroplasts involved in the toxicity of H_2O_2 and that the inactivation of SH-enzymes is not caused by H_2O_2 directly.

In contrast, addition of SOD did not prevent loss of PRK activity (Fig. 5), also suggesting that superoxide per se is not involved in oxidative inactivation of SH-enzymes. The light-mediated Fd/thioredoxin-regulating system has been removed upon isolation of thylakoids. The protective effect of mannitol on PRK in vivo would be more complex, since PRK is also subject to light-mediated regulation. DTT in our system, which starts with an active PRK in assays kept in the dark (Fig. 5), directly scavenges hydroxyl radicals (Halliwell, 1981; Spinks and Woods, 1990), mimicking the chloroplast thiol-activating machinery that protects the enzyme in vivo. This may be similar to the conditions in chloroplasts, whereby the thiol-reducing system becomes activated by light and is protected by mannitol (Shen et al., 1997). Mannitol may have protective effects on SH-enzymes and other SH-regulating chloroplast components (Fd, thioredoxin, and glutathione) during oxidative stress in the cell, considering the sensitivity of the SH-enzymes to H_2O_2 and the hydroxyl radical-scavenging ability of mannitol in chloroplasts. Furthermore, cells from transgenic tobacco plants that contained at least 100 mM mannitol in the chloroplast (Shen et al., 1997) maintained higher PRK activity when treated with H_2O_2 (1 mM) than did wild-type cells, indicating that a similar protective effect could be accomplished by mannitol accumulation in the chloroplast (B. Shen and E. Sheveleva, unpublished data). Although mannitol may have protective effects on SH-enzymes during oxidative stress, the direct in vivo evidence is still missing. Further studies are needed to elucidate the significance of this protective effect to cell function.

The mechanism of protection by mannitol is not completely understood. Although polyols might chemically

react and thus remove hydroxyl radicals that would affect PRK, the final products of such a reaction between mannitol and hydroxyl radicals in the chloroplast may be highly variable. Reactions with hydroxyl radicals can be classed into three main types: hydrogen abstraction, addition, and electron transfer (Halliwell and Gutteridge, 1995). Hydrogen abstraction by hydroxyl radicals with mannitol forms water and generates an unpaired electron on one of the C atoms, usually C1 or C6, forming a mannitol radical with an aldehyde function able to react with thiobarbituric acid. In the presence of oxygen this unstable mannitol radical converts to Man (Franzini et al., 1994). The primary mannitol radical may also undergo bimolecular disproportionation to form ketones or dimerize (Moore et al., 1979). Such radicals may also undergo elimination of water prior to the disproportionation and dimerization reactions to form deoxyketones and deoxyketo-dimers.

Compared with enzymes of the Calvin cycle, including the three SH-enzymes, PRK, sedoheptulose bisphosphatase, and Fru bisphosphatase, photosynthetic electron transport has been shown to be less sensitive to ROS (Asada, 1994). At 0.5 mM H_2O_2, for example, photosynthetic electron transport activity was not significantly inhibited, whereas this concentration inhibited CO_2 fixation by more than 90% (Asada and Takahashi, 1987). Scavengers of hydroxyl radicals could only partially prevent photoinactivation of PSI and PSII, usually less than 35% (Tschiorsch and Ohmann, 1993; Jakob and Heber, 1996), suggesting that hydroxyl radicals are not a primary damaging agent of the light-harvesting complexes, photosystems, and electron carriers. This was confirmed by observations (data not shown) indicating that mannitol and sodium formate do not show any significant protective effects on PSII activity in the isolated thylakoids. Superoxide, in addition, was not involved in photoinhibitory damage of PSII in spinach thylakoids (Hideg et al., 1995). Thus, we propose that the SH-enzymes of the Calvin cycle and the other SH-components, rather than the photosynthetic electron transport machinery, are the primary targets of superoxide and hydroxyl radicals.

ACKNOWLEDGMENTS

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